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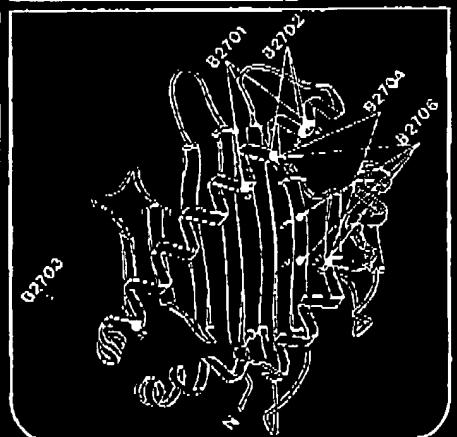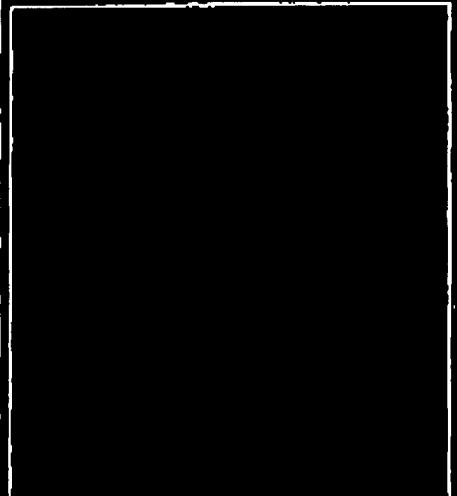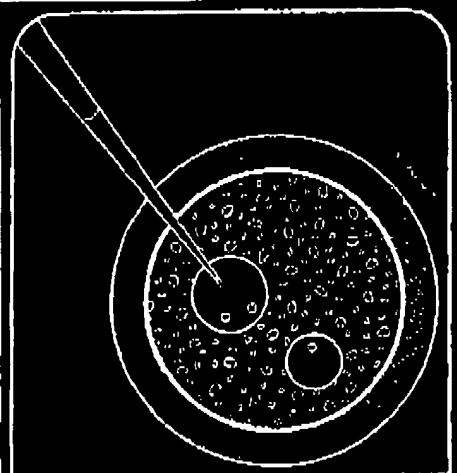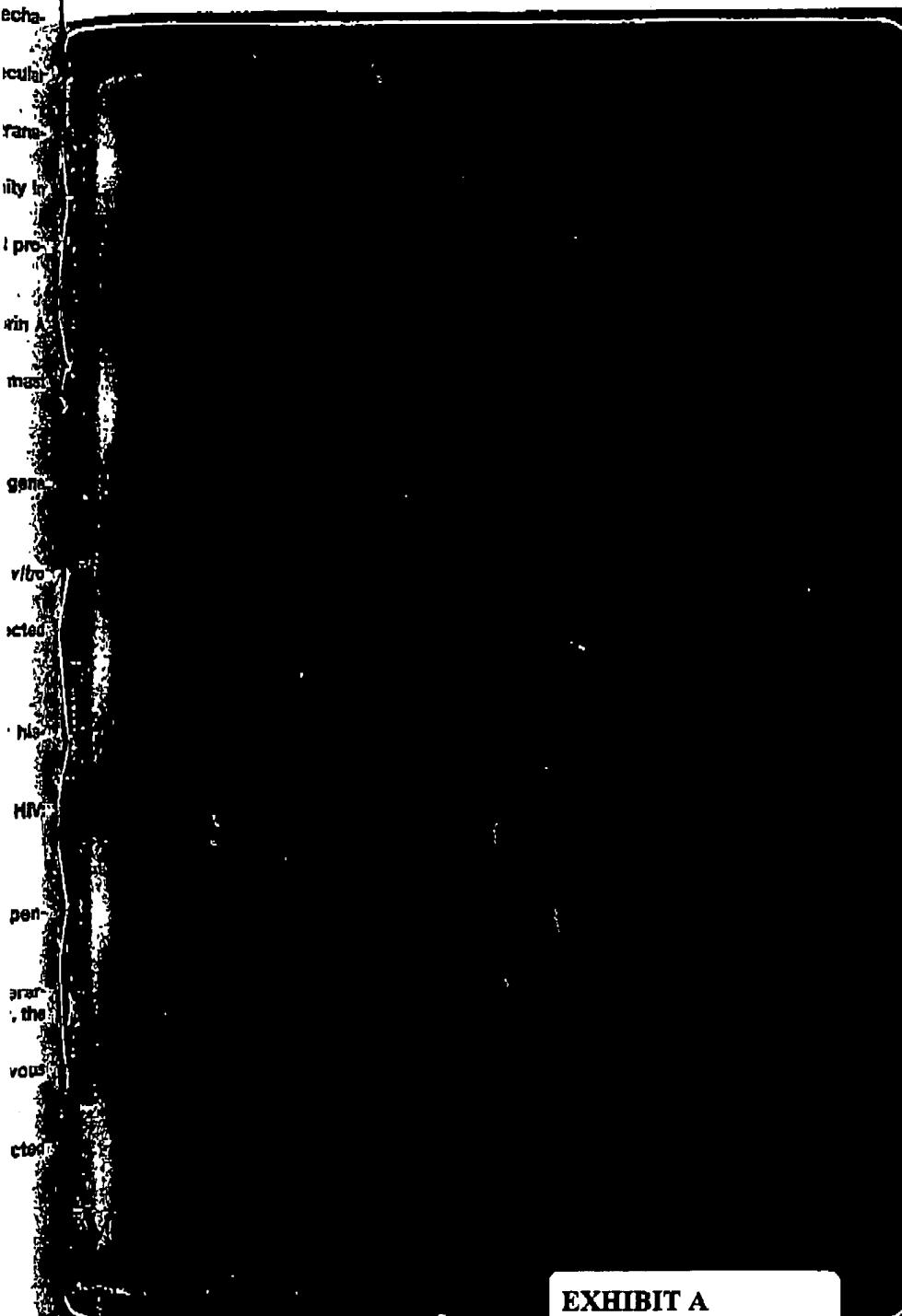
EXHIBIT A

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Mapping of murine IgE epitopes involved in IgE-Fcε receptor interactions*

The generation of anti-IgE monoclonal antibodies has permitted the identification of various serological epitopes on the IgE molecule. The relationship of the sites on IgE recognized by such antibodies to the Fc epsilon receptor (FcεR) interaction site has been determined using cross-inhibition studies. However, interpretation of this type of experiment is limited by problems of steric hindrance. Thus, to accomplish precise mapping on the IgE molecule of the FcεR interaction site and the binding sites of various anti-IgE mAb, we employed site-directed mutagenesis of the IgE heavy chain gene.

To this end we have constructed and expressed a recombinant murine constant ε heavy chain ($C_ε$) gene bearing a (4-hydroxy-3-nitrophenyl)acetic acid (NP)-binding V_H region. Several site-specific mutants in the $C_ε 3$ and $C_ε 4$ domains of this recombinant $C_ε$ gene were prepared and expressed by transfection into the light chain-producing J558L myeloma cell line. The resulting IgE antibodies were tested for binding to mast cells and to various anti-IgE mAb.

The mutants produced include a proline to histidine point mutant at amino acid residue 404 in the $C_ε 3$ domain, a mutant with a truncated $C_ε 4$ domain, a mutant with a 45 amino acid deletion in the carboxy end of $C_ε 3$, and a chimeric human $C_ε$ in which the human $C_ε 3$ was replaced by the homologous mouse $C_ε 3$ domain. These mutants have permitted the localization, to the $C_ε 3$ domain, of the epitopes recognized by the 84.1C and 95.3 anti-IgE mAb. The 84.1C mAb recognizes a site on IgE which is identical or very close to the FcεR binding site, and 95.3 recognizes a site on IgE which is related, but not identical to the FcεR binding site. The antigenic determinant recognized by the 51.3 mAb, which is inefficient at blocking the IgE-FcεR interaction, has been mapped to the $C_ε 4$ domain. When tested for binding to the FcεR on RBL-2H3 cells, the point mutant bound to the FcεR with twofold reduced affinity, while the $C_ε 3$ deletion mutant and the mutant truncated in $C_ε 4$ lost all receptor binding activity. These data suggest that the FcεR binding site can be assigned to the third C region domain, and that the fourth domain, while not directly involved in FcεR binding, may play a role in the formation of the H_2L_2 tetrameric IgE molecule, and in stabilizing the conformation of IgE required for FcεR binding.

1 Introduction

IgE antibodies are the class of antibody responsible for mediating the allergic response. Mast cells bear an Fcε receptor (FcεR) which is able to bind to IgE with high affinity. Antigen binding causes the cross-linking of the mast cell-bound IgE, triggering a series of responses in the mast cell leading to degranulation and the release of histamine and other mediators of allergy.

[I 7408]

Much work has been done in an attempt to identify the site on the IgE molecule which interacts with the FcεR. Most of the data available to date implicate the third constant (C) region domain, and the interface between the second and the third domain of IgE, in receptor binding. Experiments by Pérez-Montfort and Metzger [1] have shown that a region in the cleft between the second and third C region domains is protected from proteolysis when bound to the FcεR. Burt et al. [2, 3] made synthetic peptides corresponding to regions of the rat IgE molecule thought to be accessible to the external environment. One of these peptides, P129, corresponding to residues 414–428 in the third C region domain ($C_ε 3$), (residues 401–415 using the numbering system of Liu [4], the convention adopted in this report) inhibits the binding of IgE to the FcεR on a rat mast cell line (RBL-2H3). The binding of P129 is about 1000-fold less efficient on a molar basis than the binding of intact IgE.

In an analogous set of experiments, Robertson and Liu [5] have prepared antisera against peptides corresponding to regions of the mouse second, third, and fourth C region domains. Most of these antisera reacted against native IgE and showed varying reactivities against mast cell-bound IgE. One anti-peptide antibody, made against a peptide from $C_ε 3$, "E-peptide-3", bound well to free IgE, but not to cell-bound IgE. This peptide may therefore represent a site sterically close to the epitope on IgE recognized by the FcεR. Interestingly, anti-

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sera raised by Robertson and Liu against a peptide overlap-
ping (by eight residues) Stanworth's P129 peptide, were able
to bind cell-bound IgE. This led them to the conclusion that
the site corresponding to this peptide is not involved in $Fc_\epsilon R$
binding.

Helm et al. [6] have generated recombinant peptides contain-
ing stretches of the human $C_\epsilon 2$, $C_\epsilon 3$ and $C_\epsilon 4$ domains. They
found that a peptide containing 76 amino acids spanning the
$C_\epsilon 2$-$C_\epsilon 3$ junction is able to bind to the $Fc_\epsilon R$ on mast cells with
an affinity close to that of myeloma IgE. In addition, mono-
meric fragments were produced which still bound to the $Fc_\epsilon R$,
indicating that a heavy (H) chain dimer is not necessary for
$Fc_\epsilon R$ binding. Nevertheless, it is not clear whether an analo-
gous region at the $C_\epsilon 2$-$C_\epsilon 3$ junction, containing the $Fc_\epsilon R$ bind-
ing site, is present on rodent IgE.

A number of anti-IgE monoclonal antibodies (mAb) have
been generated in our laboratory [7, 8]. 84.1C, 95.3 and 51.3
define three separate groups of determinants on the C_ϵ region.
All three of these mAb block, to varying degrees, the binding
of IgE to the $Fc_\epsilon R$ on mast cells. However, only the 84.1C
antibody is unable to bind to cell-bound IgE. This suggests
that 84.1C recognizes an epitope identical to, or closely associ-
ated with the $Fc_\epsilon R$ binding site on IgE.

In order to reconcile some of the data on IgE-$Fc_\epsilon R$ interaction
site (s), and to identify the sites on the IgE molecule recog-
nized by our anti-IgE mAb, we constructed a panel of IgE
mutants. Testing of these mutant IgE for their ability to bind
to mast cells and to our anti-IgE mAb, should help create a
map of various epitopes on the murine IgE molecule involved
in the $Fc_\epsilon R$ binding.

2 Materials and methods

2.1 Plasmids and vectors

The pSV2-V_H6 expression vector (Fig. 1A) contains the com-
plete rearranged V_H gene of an anti-NP (4-hydroxy-3-ni-
trophenyl)acetic acid antibody of the NP^b family [9], a gift of
Dr. Doug Rice. It is based on the pSV2gpt vector [10], a
mammalian expression vector containing selectable markers
for growth in both bacteria (β-lactamase gene) and mamma-
lian cells (gpt gene). The pA-6 vector consists of the pBR322
plasmid into which a mouse rearranged genomic IgM H chain
was introduced. The pBR-C_ϵ plasmid [11], containing a
genomic clone of the mouse IgE H chain cloned into the
pBR322 plasmid, was a kind gift of Dr. T. Honjo (Fig. 1B).

2.2 Molecular biology techniques

Plasmid preparations, digestions with restriction enzymes,
separation of DNA fragments, ligations, bacterial transforma-
tions and screening of bacterial colonies were all performed by
standard techniques [12]. Restriction enzymes and DNA mod-
ifying enzymes were purchased from New England BioLabs
(Beverly, MA), Bethesda Research Labs (Bethesda, MD),
IBI (New Haven, CT), and Pharmacia (Uppsala, Sweden).
Sequencing reactions were carried out by the dideoxy method,
after subcloning into the pGEM-3 vector, using the kit sup-
plied by Promega (Madison, WI).
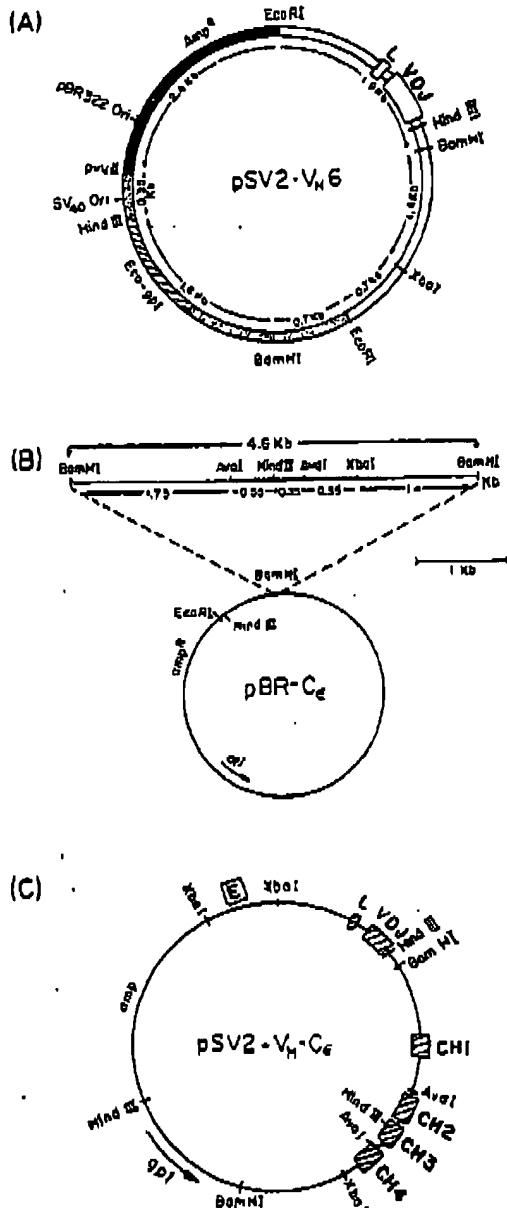
Figure 1. Schematic diagram of: (A) the pSV2-V_H6 plasmid based on
the pSV2gpt vector [10], into which the rearranged VDJ genomic
segment encoding an anti-NP [9] V_H chain was inserted. (B) The
pBR-C_ϵ plasmid containing a segment of genomic DNA encoding all
four C_ϵ exons [11] inserted into the pBR322 plasmid. (C) The pSV2-
V_H-C_ϵ plasmid, composed of the pSV2-V_H6 vector (A), into which the
C_ϵ gene (from pBR-C_ϵ) and an Ig enhancer sequence [E] were inserted
flanking the rearranged VDJ segment.

Site-specific mutagenesis was carried out by heteroduplex for-
mation as described [13] with slight modifications. An aliquot
of the PBR-C_ϵ plasmid was digested with SalI, and blunt-
ended; a second aliquot was digested with Eco RV and the 6.7-
kb band was purified. Approximately 100-200 ng of each frag-
ment was mixed in high-salt restriction buffer [12] together
with 8 pmol kinased mutagenized oligomer (see Sect. 3 for
sequence), boiled for 3 min and allowed to gradually reanneal.
Heteroduplexes were filled in whith DNA polymerase and
ligase, and the resulting product was extracted with phenol,

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ethanol precipitated and used to transform competent bacteria (MC1061 strain).

2.3 Transfections and cell culture

RBL-2H3 cells [14], obtained from Drs. D. and T. Jovin, were grown in minimum Eagle's medium with the addition of 10% fetal calf serum (BioLab Ltd., Jerusalem, Israel), L-glutamine (Biological Industries, Kibbutz Beth Haemek, Israel), combined antibiotics (BioLab Ltd.) and fungizone (amphotericin, Biological Industries). J558L myeloma cells [15] (a gift of Dr. V. T. Oi), were grown in RPMI medium with 10% fetal calf serum (FCS), L-glutamine, and combined antibiotics (referred to as J558L medium).

Transfections were carried out by electroporation [16] or protoplast fusion [17]. For electroporation, 20 µg of purified plasmid was mixed with 2×10^7 J558L cells in phosphate-buffered saline (PBS) and subjected to a 1.8 kV discharge with an Isco (Nebraska, CO) power supply model 494. Cells were put into regular J558L medium for 24–48 h reovery and then transferred to selective medium.

Protoplast fusion was done as described [17]. Protoplasts prepared from 25-ml bacterial culture, containing the plasmid of interest were fused with polyethylene glycol (PEG) 1550 (Serva, Heidelberg, FRG) to 2×10^7 J558L cells. The cells were plated in 24-well plates at 10^5/ml and cultured in J558L medium in the presence of antibiotics [100 µg/ml kanamycin or gentamycin (Sigma, St. Louis, MO)], to kill any remaining viable bacteria. Selective medium was added at 24–48 h and clones were visible in 2–3 weeks. Selective medium contained regular J558L medium with the addition of 1 µg/ml mycophenolic acid, 250 µg/ml xanthine and 15 µg/ml hypoxanthine (Sigma).

2.4 Immunochemical reagents

Rabbit anti-mouse IgE was made in our laboratory by immunizing rabbits with several mouse mAb of the IgE class. Antisera were preadsorbed on normal mouse serum-coupled Sepharose and then further purified on an IgE-Sepharose affinity column. These antibodies recognize the C_ε chain and also react with λ1 light (L) chains which are present on the SPE-7 monoclonal IgE used for immunization. Biotinylation was done by the method of Kendall et al. [18]. NIP-protein conjugates were made by the method of Brownstone et al. [19] and coupled to Sepharose 4B following cyanogen bromide activation.

2.5 IgE purification

Lysates were produced from non-secreting mutants (AFE), by suspending cells in PBS, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (Sigma), 5 mM iodoacetamide and 0.2 U/ml aprotinin (Sigma). Cell suspensions were lysed either by freeze-thawing, sonication or the addition of 1% Nonidet-P40 (NP40).

Anti-NIP [(4-hydroxy-3-iodo-5-nitrophenyl)acetate] IgE antibodies were purified on NIP-ovalbumin (OVA)-Sepharose columns. J558L transfectoma supernatant (200–500 ml) or 10 ml of cell lysate from 5×10^8 cells, were passed over NIP-OVA-Sepharose columns. The columns were then washed with PBS containing 0.5% NP40 and then extensively washed with PBS. Elution was carried out with 5 M MgCl$_2$. Eluates were dialyzed extensively against PBS, aliquoted and frozen.

2.6 Immunoassays for IgE anti-NIP antibodies

IgE anti-NIP antibodies were detected by one of several variations of the enzyme-linked immunosorbent assay (ELISA) or radioimmunoassays (RIA) described here. For the RIA, 96-well polyvinylchloride V-shaped wells (Cooke Labs., Alexandria, VA) were coated with 50 µl antigen solution (10 µg/ml NIP$_5$DNP$_6$OVA), or anti-IgE, overnight at 4°C. Plates were washed with PBS containing 0.1% bovine serum albumin (BSA), and blocked with PBS containing 1% BSA to reduce nonspecific binding. IgE preparations (usually 50 µl transfectoma supernatant) were then added for 2 h at room temperature, followed by ^{125}I-labeled anti-IgE. Wells were washed, cut apart and counted in a gamma counter.

For ELISA assays, flat-bottom ELISA plates (Nunc, Roskilde, Denmark) were coated with either 10 µg/ml NIP-fowl γ-globulin (FgG), or a 1:10 dilution of serum-free supernatant from hybridoma cells secreting anti-mouse IgE mAb [7, 8], diluted in 0.06 M sodium carbonate buffer, pH 9.5. After overnight incubation at 4°C, the plates were washed in PBS containing 0.05% Tween, and dilutions of IgE-containing transfectoma supernatants, or purified antibody were added, and the plates were incubated for 2 h at 37°C. Plates were then washed and a 1:500 dilution of biotinylated rabbit anti-mouse IgE was added for 2 h. After washing, plates were treated for 30 min with avidin-coupled peroxidase (Bio-Makor, Rehovot, Israel), washed and peroxidase substrate was added. Alternatively, for the anti-NIP ELISA, after incubation with the IgE samples, plates were treated with a 1:1000 dilution of peroxidase-conjugated anti-mouse Fab (Bio-Makor). Peroxidase substrate was added after washing. The substrate used was 2,2'-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) (Sigma), dissolved at 1 mg/ml in 28 mM citric acid, 44 mM Na$_2$HPO$_4$, 0.3% H$_2$O$_2$. Assays were read in an automatic ELISA reader (Titertek Multiskan; Flow Labs., Rockville, MD) at 620 nm.

2.7 Binding of iodinated IgE or RBL-2H3 cells

Purified IgE derived from transfectomas was iodinated using the chloramine-T method. Protein (40–100 µg) in 100 µl PBS was reacted with 0.5–1.0 mCi (= 18.5 37 MBq) of ^{125}I and 10 µl of chloramine T (2 mg/ml) for 2 min. The reaction was stopped with 20 µl of sodium disulfide (2 mg/ml) and the mixture chromatographed on a 7-ml Sephadex G-25 column in the presence of 1% BSA, KI, and hemoglobin solution to color the protein peak. The first radioactive peak was collected, and incorporation of iodine checked by trichloroacetic acid precipitation.

For binding studies, 10^6 RBL-2H3 cells/tube were mixed with dilutions of radiolabeled IgE, in 250 µl Tyrode's buffer [20] with 1 mM EDTA. For competition assays, cold IgE competitor was added and the mixture incubated for 2 h before the addition of the radiolabeled IgE. After an additional 2-h incubation with shaking at 4°C, 50-µl aliquots were removed in

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triplicate and layered over horse serum in microtubes. The tubes were centrifuged in a microfuge for 30 s, supernatant was aspirated and pellets were counted in a gamma counter. An additional 50-μl aliquot from each sample was counted to determine total counts added. Direct binding data were analyzed by Scatchard analysis and linear least square regression.

2.8 Degranulation assay

RBL-2H3 cells (10^6) were incubated with each IgE sample for 30 min at 37°C. Cells were washed with Tyrode's buffer and resuspended in 1 ml Tyrode's buffer. Aliquots (100 μl) were placed in microtiter wells to which were added either 250 ng/ml NIP-FgG to measure specific release or 15 μl 10% Triton to measure maximum release, or nothing to measure spontaneous release. The plates were incubated for 30 min at 37°C and then 15 μl of supernatant was removed from each well to measure β-hexosaminidase release, an indicator of degranulation. The supernatants removed were mixed in wells of an ELISA plate with 40 μl substrate solution [1.3 mg/ml paranitrophenyl-N-acetyl-β-D-glucosamine (Sigma) in 0.04 M phosphate/citrate buffer, pH 4.5] and incubated 1–2 h at 37°C. To stop the reaction 0.2 M glycine, pH 10.7, was added and wells were read in an ELISA reader at 405 nm. Percent specific release was calculated as:

$$\frac{(\text{specific release} - \text{spontaneous release})}{(\text{maximum release})} \times 100,$$

where spontaneous release is release in the absence of antibody, and maximum release is the β-hexosaminidase activity obtained after 1% Triton lysis of cells.

2.9 Adsorption assay for binding to the $Fc_{\varepsilon}R$

As an alternative means to determine the extent of binding of the mutant IgE to the $Fc_{\varepsilon}R$ on mast cells, we measured the ability of RBL-2H3 cells to remove anti-NIP activity from IgE-containing preparations. Dilutions (100 μl) of purified mutant and wild-type IgE were incubated in triplicate in microtiter wells with either 100 μl PBS or 100 μl PBS containing 10^6 RBL-2H3 cells. The plates were incubated for 3–4 h at room temperature, with periodic shaking. Supernatant (100 μl) was then removed from each well, and assayed for anti-NIP activity by ELISA, on NIP-FgG-coated ELISA plates. The ratio of optical density obtained from the ELISA from IgE preparations incubated with RBL-2H3 cells vs. those incubated without RBL-2H3 cells gives a measure of how much IgE was removed by these cells, and thereby indicates to what extent the IgE was able to bind to these cells.

2.10 Western blots and immunodetection

IgE transfectoma lysates, cell supernatants, or purified IgE preparations were run on sodium dodecyl sulfate (SDS) polyacrylamide gels [21]. After electrophoresis proteins were transferred to nitrocellulose in 15.6 mM Tris, 120 mM glycine, pH 8.3 for 4 h at 200 mA. The nitrocellulose filters were stained with Ponceau red (Sigma) to visualize protein standards, prehybridized in PBS containing 10% skim milk for 16 h at 4°C and then hybridized in the above buffer with ^{125}I-labeled rabbit anti-mouse IgE. Filters were washed in PBS and PBS-Tween and autoradiographed.

3 Results

3.1 Construction of the PSV2-V_H-C_ε plasmid

In order to generate IgE molecules with various mutations in the Fc portion of their C region, we constructed a vector which permitted the expression, in a mammalian cell, of a complete IgE molecule of known antigenic specificity. This was accomplished by joining the gene segment from a genomic clone encoding the mouse C_ε region with a V_H gene fragment encoding a V region with anti-NP specificity [9]. The V region encoded by this gene is heteroclitic and binds the antigen NIP with higher affinity than NP. All assays measuring hapten binding were therefore performed using NIP-conjugated rather than NP proteins. The PSV-V2$_H$-C_ε plasmid which we constructed (see below) contains the entire mouse C_ε coding sequence and an anti-NP variable region (Fig. 1C). Upon transfection into the L chain-producing J558L myeloma cell line, the ε chain encoded by the recombinant gene combines with the endogenous J558L L chain to produce an IgE antibody with anti-NP specificity. Similar constructs have been used previously by Neuberger et al. [22] to obtain chimeric IgE molecules bearing a human ε gene and a NP-binding mouse V region.

The PSV2-V_H-C_ε plasmid was constructed from the PSV2-V_H6 vector ([9] and Fig. 1A). This expression vector contains a rearranged V_H gene of anti-NP specificity [9]. The 6.25-kb fragment obtained after Bam HI digestion was ligated to the 4.6-kb Bam HI fragment of the PBR-C_ε plasmid (Fig. 1B), containing the full mouse C_ε region [11]. The vector so obtained (denoted PSV2-C_ε-E) contained the full C_ε and V_H segment but was lacking the enhancer element for Ig transcription. In order to increase the expected levels of Ig expression with this vector, the 1-kb fragment containing Ig enhancer activity, obtained fom XbaI digestion of the pA-6 plasmid, was inserted into the unique EcoRI site of the PSV2-C_ε-E vector by blunt-end ligation of filled-in ends. The vector thus obtained was called PSV2-V_H-C_ε and its structure is shown in Fig. 1C.

The PSV2-V_H-C_ε plasmid was introduced into J558L L chain-producing myeloma cells using the technique of protoplast
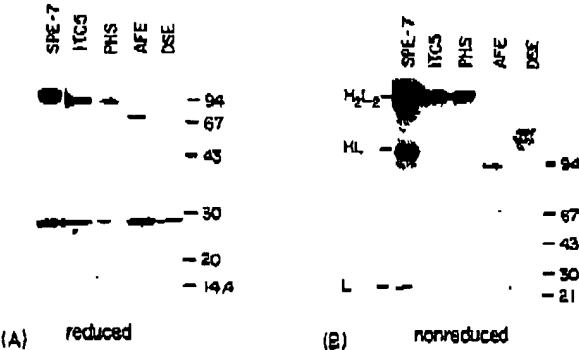

Figure 2. Immunoblot analysis of mutant and wild-type IgE antibodies. Purified IgE preparations (25–100 ng/lane) were run under reducing (A) or nonreducing (B) conditions through 10% or 5%–10% gradient acrylamide gels, respectively, electroblotted to nitrocellulose and hybridized with iodinated rabbit anti-mouse IgE antibodies. Note that in addition to the ε chain, the anti-IgE antibodies cross-react with the λ L chain.

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Table 1. Properties of ITCS recombinant IgE

a) Mouse monoclonal IgE [23].
b) Determined by Scatchard analysis of binding of radioiodinated IgE to RBL-2H3 cells.
c) Concentration of IgE giving 50% inhibition of the binding of radioiodinated SPE-7 to RBL-2H3 cells.
d) % RBL-2H3 cell degranulation induced by IgE at 100 ng/ml, triggered by NIP-DNP-BSA.

fusion. Supernatants from clones surviving growth in selective medium containing mycophenolic acid were screened for IgE anti-NIP activity using a RIA (not shown) and the ITC5 clone was chosen for all further characterization. This clone secretes approximately 2 µg IgE/ml. Table 1 summarizes properties of the ITC5 IgE as compared to SPE-7 hybridoma-derived monoclonal IgE [23]. Both IgE have similar molecular weights (Fig. 2), bind with similar affinities to the Fc$_\epsilon$R on mast cells and both are able to induce RBL-2H3 degranulation in the presence of cross-linking antigen.

3.2 Generation of vectors encoding mutant IgE antibodies

The mutated IgE antibodies that we describe in this study are depicted schematically in Fig. 3 and Table 2. They include AFE, truncated in C$_\epsilon$4, DSE, with a 45-amino acid deletion in the C$_\epsilon$3 domain, and PHS, with a proline to histidine point mutation in C$_\epsilon$3. The AFE mutant was made by creating a frameshift mutation at the Ava I site in the C$_\epsilon$4 domain (Fig. 1B, C). This frameshift caused a stop codon to be reached at amino acid 472 (position 34 of the C$_\epsilon$4 domain). The region between the mutation site and stop codon was expected to have altered sequence due to the frameshift introduced (Fig. 3).

To generate this mutation, the PSV2-V$_H$-C$_\epsilon$ vector was partially digested with Ava I and the 11.85-kb linearized fragment was blunt ended with the Klenow fragment of DNA polymerase I. This was self ligated and used to transform bacteria. Restriction mapping and DNA sequencing were used to confirm that the correct Ava I site was mutated (not shown). The vector containing the AFE mutation was used to transfect J558L cells. Clones surviving mycophenolic acid selection were tested by an ELISA assay for secretion of anti-NIP antibody. Of nine clones tested from two separate transfection experiments, none secreted detectable amounts of IgE. (The sensitivity of the ELISA is approximately 5 ng/ml). It is possible that the cells can produce IgE, but that due to the gross structural alteration of IgE, they are unable to secrete IgE. We therefore produced cell lysates, using sonication, freeze-thawing or NP40 lysis. The lysates were tested for IgE anti-NIP activity by ELISA and were found to contain between <5 and 110 ng/10^6 cells. Immunoblot analysis (Fig. 2) under reducing conditions of affinity-purified AFE mutant IgE from such lysates revealed H chains of reduced size as expected for this mutant. Electrophoresis under nonreducing conditions reveals

that almost all of the antibody is present as HL, instead of the full H$_2$L$_2$ tetramer (Fig. 2).

Another deletion mutant (DSE) was constructed with a deletion of 45 amino acids in C$_\epsilon$3 and the beginning of C$_\epsilon$4 (amino acid residues 397-441). The deletion encompasses the sequence spanned by the P129 peptide of Burt and Stanworth [2] and also includes a cysteine which participates in the C$_\epsilon$3 intradomain disulfide bridge [4, 24]. This mutant was made by digestion of the PSV2-V$_H$-C$_\epsilon$ plasmid with Spe I and Eco 47III (each of which cut the plasmid at a single site), blunting the ends with mungbean nuclease, religating the large linear fragment and transforming bacteria with the product of this reaction. The structure of this plasmid was checked by restriction mapping and by hybridization to an oligomer which was complementary to the expected sequence of the junction site after ligation. Transfected clones were found to secrete antibody, but in very small amounts, 5-40 ng/ml.

A proline to histidine point mutation (PHS) was created at amino acid residue 404, using oligonucleotide-directed, site-

Figure 3. Amino acid sequence of the mouse C$_\epsilon$ domains and of the IgE mutants prepared in this study. Dashes (———) indicate wild-type sequence; (x) indicates deleted amino acids; (CHO) indicates glycosylation sites and (S-S) indicates intrachain disulfide bonds. S-S-(H)- and S-S-(L)- represent the interchain disulfide bonds with H and L chains. The sequence shown is based on references [4] and [23] and the one-letter amino acid code is given in [4].

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Table 2. Summary of mutants produced in this study
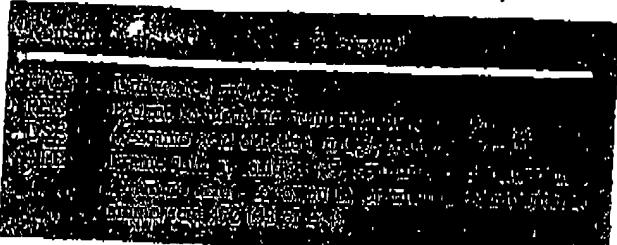
a) All the recombinant IgE molecules listed above contain a NIP-specific mouse V_H region with a C_ϵ region and express the endogenous J558L mouse λ1 L chain.

specific mutagenesis [13]. This mutation is located in $C_\epsilon 3$, in the region spanned by the P129 oligomer of Burt and Stanworth [2]. Aliquots of the PBR-C_ϵ plasmid were digested with Sal I and Eco RV, and the resulting digests were mixed, melted, and reannealed in the presence of a molar excess of kinased SAS3 oligomer (sequence GGC, AAC, TAC, ATG, TAG, GAT, GGA). This oligomer is complementary to the sequence encoding amino acids 401–407, with a C to A mutation which alters proline 404 to histidine. The oligomer also contains a silent mutation to increase the ease of detecting mutant vs. wild type. The positions of the two mutations are underlined. Mutant colonies were identified by hybridization with radiolabeled oligomer. The 4.6-kb insert corresponding to C_ϵ was removed by digestion with Bam HI and ligated into the PSV2-V_H-C_ϵ plasmid from which the wild-type C_ϵ sequences were removed. This plasmid (PSV-V_H-C_ϵPHS) was used to transfect J558L cells by electroporation. Supernatants from clones surviving mycophenolic acid selection were tested for IgE anti-NIP antibodies. The PHS clone selected secretes approximately 2 μg/ml of antibody. RNA was isolated from this clone and tested by Northern blot hybridization with the mutant oligomer for the presence of the mutated sequence. The oligomer hybridizes to PHS but not to ITC5 (wild-type) RNA (not shown). SDS gel electrophoresis of the IgE produced by the PHS mutant reveals H and L chains of the expected size, present as H_2L_2 in nonreduced gels (Fig. 2).

The preparation and expression of vectors containing the human C_ϵ sequences, and human-mouse chimeras will be described in a separate manuscript (A. Nissim and Z. Eshhar, in preparation).

3.3 Binding of the mutant IgE to the $Fc_\epsilon R$ on RBL-2H3 cells

In order to identify the sites on the IgE molecule involved in the interaction with the $Fc_\epsilon R$, the IgE mutants were tested for their ability to bind to RBL-2H3 cells, a rat mast cell line bearing high-affinity FcR for rodent IgE [14]. A number of different assays were used to measure binding. First, affinity-purified preparations of unmutated ITC5 and the IgE mutants were tested for their ability to inhibit the binding of iodinated, purified monoclonal IgE (SPE-7) [24] to RBL-2H3 cells. The inhibition curves so obtained are shown in Fig. 4A. It may be seen that the PHS point mutant is reduced approximately twofold in its ability to bind to the $Fc_\epsilon R$, while the DSE mutant has lost all ability to compete with radioiodinated IgE for the binding to RBL-2H3 cells. The AFE mutant, which is not secreted, and was therefore purified from cell lysates, was not

available in sufficient concentrations for the inhibition assay. It was therefore tested in the other binding assays described below. Since the differences in the inhibition of binding by the ITC5 wild-type IgE and the PHS mutant were small, these IgE preparations were also tested for direct binding to RBL-2H3 cells. Affinity-purified ITC5 and PHS were radioiodinated and the binding of these antibodies to RBL-2H3 cells was tested. Scatchard analysis (Fig. 4B) reveals that the PHS mutant has a 2.5-fold lower affinity for the $Fc_\epsilon R$ than ITC5 wild-type IgE.

These results were further confirmed in two other assays. In an assay measuring the degranulation of RBL-2H3 cells, the ITC5 (wild type) and PHS (mutant) IgE antibodies, but not the DSE or AFE mutant IgE were able to induce degranulation of RBL-2H3 cells, when cross-linked on the cell surface by polyvalent antigen (NIP-FgG) (Table 3).


(A)

(B)
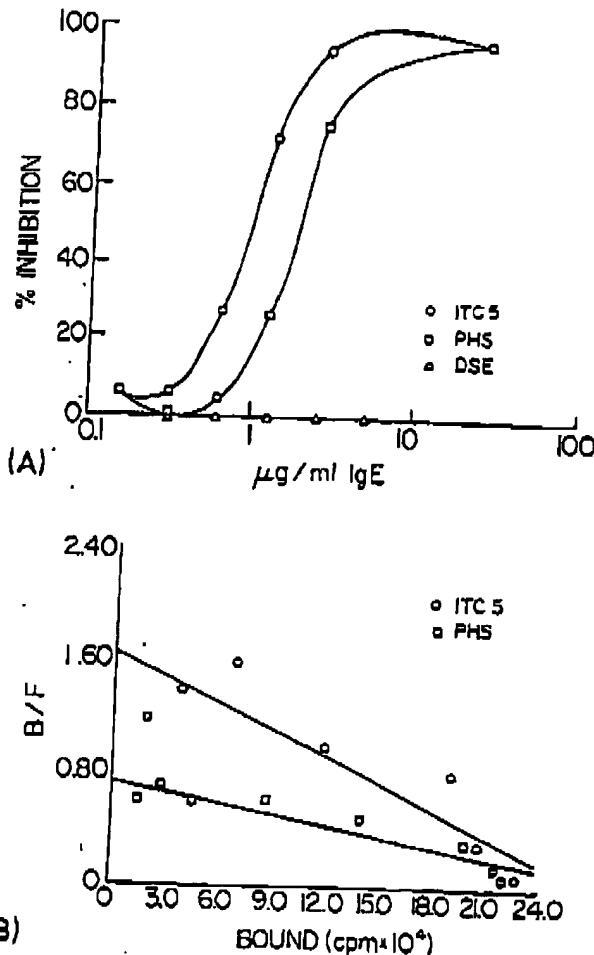
Figure 4. $Fc_\epsilon R$ binding of mutant IgE antibodies. (A) Inhibition of binding of iodinated IgE to RBL-2H3 cells by mutant and wild-type recombinant IgE. RBL cells were preincubated with varying concentrations of ITC5 (wild type), PHS (point mutant) or DSE (deletion mutant) IgE. ^{125}I-labeled monoclonal IgE was then added and after a 2-h incubation, cell-bound and free IgE were separated and radioactivity was counted. All determinations were done in triplicate. (B) Scatchard analysis of binding of ITC5 (unmutated) and PHS, point mutant IgE. RBL-2H3 cells were incubated with several concentrations of iodinated ITC5 and PHS IgE. Unbound counts were removed by centrifugation through serum, and the cell pellet was counted. Lines represent the least squares fit, derived by linear regression analysis.

Table 3. Degranulation of RBL-2H3 cells mediated by IgE mutants

a) RBL-2H3 cells were preincubated with affinity-purified IgE preparations at 0.2 μg/ml for 30 min and washed before the addition of NIP-FgG (250 ng/well). β-Hexosaminidase release was determined as described in Sect. 2.8. Each determination was done in triplicate. Standard deviations were < 15% of ELISA readings.

Finally, mutant IgE antibodies were tested for their ability to be adsorbed by RBL-2H3 cells. Dilutions of the affinity-purified antibodies were incubated with RBL-2H3 cells to allow binding. Supernatants containing unbound IgE were tested by ELISA for anti-NIP activity. Results were plotted as the ratio between the ELISA readings obtained for adsorbed supernatants, divided by unadsorbed supernatants, vs. the concentration of the IgE preparation used (represented by ELISA reading). As can be seen in Fig. 5, the ratio of A_{620} "adsorbed"/A_{620} "unadsorbed" was lowest for ITC5, the wild-type IgE (best adsorbed), and higher for the PHS mutant, though some anti-NIP activity was still removed. For the AFE and DSE mutants, the ratio was close to one, indicating that the RBL-2H3 cells did not remove any of the IgE anti-NIP antibodies. Thus, we may conclude that binding of mutant IgE molecules to the $Fc_\epsilon R$ on rat mast cells follows the order: ITC5 > PHS ≫ AFE = DSE.

3.4 Recognition of mutant IgE antibodies by anti-IgE mAb

Purified mutant and wild-type IgE antibodies were next tested for their ability to bind to anti-mouse IgE mAb which have been previously described [7, 8]. ELISA assays were performed in which wells were coated with either antigen (NIP-FgG), or serum-free preparations of the anti-IgE mAb. This was followed by dilutions of purified wild-type and mutant IgE preparations. Degree of binding was determined using biotiny-

lated rabbit anti-mouse IgE (H + L), and peroxidase-labeled avidin. Table 4 shows the results of one representative assay.

It may be seen that the PHS point mutant has lost the ability to be recognized by the 95.3 anti-IgE mAb, while it is still recognized by the 51.3 and 84.1C mAb (Table 4) and can still bind to mast cells (though with reduced affinity) (Fig. 4). The DSE deletion mutant has lost both recognition by all three mAb and the ability to bind to the $Fc_\epsilon R$ on mast cells. The AFE mutant which has a frameshift causing premature termination in the $C_\epsilon 4$ domain has reduced binding to the 51.3 anti-IgE mAb, and has lost the ability to bind to the FcR on RBL-2H3 cells. A chimeric human IgE ($H_\epsilon MC_\epsilon 3$) containing mouse $C_\epsilon 3$ (manuscript in preparation), binds the 84.1C and 95.3 mAb, but not the 51.3 mAb. "TAN" human IgE (manuscript in preparation), with the J558L L chain and mouse anti-NIP V_H region, is not recognized by any of the mAb. These data permit the assignment of the site recognized by 84.1C and 95.3 to the $C_\epsilon 3$ domain, and that recognized by 51.3 to the $C_\epsilon 4$ domain.

4 Discussion

In order to learn more about the $Fc_\epsilon R$ binding site on the IgE molecule, we have developed an in vitro expression system enabling the production of genetically engineered mouse IgE with anti-NP activity. The antibody so produced, represented by clone ITC5, has all the properties of authentic mouse IgE and binds with high affinity to the $Fc_\epsilon R$ on mast cells (Table 1). The plasmid encoding the ITC5 antibody was then subjected to a variety of mutagenesis procedures in order to produce IgE molecules with alterations in their Fc region. These mutants were used in binding assays to determine their ability to bind to the $Fc_\epsilon R$ on mast cells and to bind to a series of anti-IgE mAb previously generated in our laboratory [7, 8].

Four assays were employed to measure the binding of mutant or wild-type IgE to the $Fc_\epsilon R$ on mast cells: (a) the inhibition of binding of radiolabeled IgE to RBL-2H3 cells, (b) the direct binding of iodinated IgE to RBL-2H3 cells, (c) IgE-mediated binding of RBL cells and (d) the adsorption, by RBL-2H3 cells, of anti-NIP activity from IgE-containing solutions. These assays gave concordant results. Compared to the ITC5 wild-type IgE, the PHS proline to histidine point mutant bound the $Fc_\epsilon R$ with approximately twofold reduced affinity (Figs. 4A, B and 5). PHS mutant IgE induced almost as much degranulation as did ITC5 wild-type IgE (Table 3). Since only

Table 4. Binding of IgE mutants to anti-IgE mAb

a) Purified unmutated (ITC5) and mutated recombinant IgE were tested by ELISA for binding to various anti-mouse IgE mAb. ELISA plates were first coated with serum-free supernatant containing the various anti-IgE mAb, or with NIP-conjugated protein. IgE (4 ng) diluted in medium (DMEM and FCS) was then added to each well. After washing, the wells were treated with biotin-conjugated rabbit anti-mouse IgE, followed by an avidin-peroxidase conjugate and peroxidase substrate. Absorbance was read in an automatic ELISA reader. Variation between duplicates was always < 10% of the values read, or < 0.01 absorbance units for negative wells.
b) Anti-mouse IgE mAb.
c) See Table 2.
d) Mouse $C_\epsilon 3$ into human $C_\gamma 1,2,3$.
e) Human IgE.
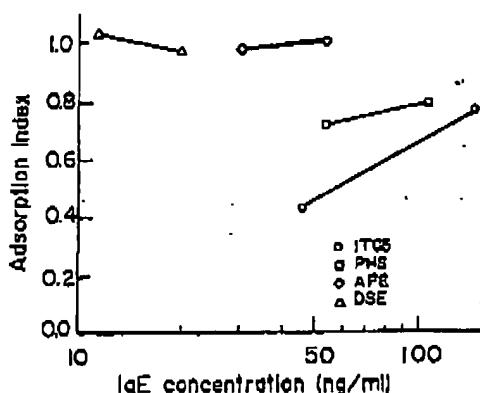
Figure 5. Adsorption of IgE by RBL-2H3 cells. Dilutions of mutant and wild-type IgE antibodies were incubated with RBL-2H3 cells for 4 h in microtiter wells. After this incubation, supernatants were removed and tested by ELISA for anti-NIP activity. Results were plotted as the adsorption index (the ratio between the ELISA readings obtained for adsorbed divided by unadsorbed supernatants) vs. the concentration of the IgE preparation tested. An adsorption index closer to unity indicates less adsorption of IgE by RBL-2H3 cells, and hence, less binding of the particular mutant.

a small fraction of Fc$_\epsilon$R on RBL-2H3 cells need to be occupied in order to induce degranulation, it is not surprising that sufficient occupancy could be obtained with either ITC5 wild type or PHS point mutant IgE.

The AFE frame shift mutant, lacking a functional C$_\epsilon$4 domain, and the DSE mutant, with a 45-amino acid deletion in C$_\epsilon$3, are unable to bind to the Fc$_\epsilon$R in the four assays used (Figs. 4 and 5 and Table 3). It is surprising, in light of the accumulated evidence [1–3, 5] suggesting that the binding site for the Fc$_\epsilon$R is present on C$_\epsilon$3, that the AFE mutant (lacking the C$_\epsilon$4 domain) is unable to bind to the Fc$_\epsilon$R. We believe that the C$_\epsilon$4 domain, while not containing the actual receptor binding site(s), is necessary for the IgE molecule to attain the correct conformation needed for receptor binding. The C$_\epsilon$4 domain has been shown to be important for the dimerization of human IgE [6]. Interestingly, our AFE mutant mouse IgE which lacks most of the C$_\epsilon$4 domain, is present inside the cell almost entirely as HL (Fig. 2). This indicates that in mouse IgE, the fourth domain may also have a role in initiating, or stabilizing H chain dimerization.

The epitopes recognized by three anti-IgE mAb, 84.1C, 95.3 and 51.3, were assigned to domains on the ε chain based on their ability to recognize the various IgE mutants tested. For example, the 95.3 mAb was shown to recognize a determinant on the C$_\epsilon$3 domain, since it is no longer recognized by the PHS mutant, which has a single proline to histidine substitution in C$_\epsilon$3, and is still recognized by the AFE mutant which has 96% of its C$_\epsilon$4 domain altered or deleted. In addition, 95.3 is able to recognize the mouse-human chimeric IgE, which has only mouse C$_\epsilon$3, while it is unable to recognize human IgE. By analogous logic, the epitope recognized by 84.1C. may be assigned to C$_\epsilon$3, and the epitope recognized by 51.3 is most likely on C$_\epsilon$4 (Table 4).

The purified DSE mutant (45-amino acid deletion in C$_\epsilon$3) seems to bind none of the anti-IgE mAb, including the 51.3 antibody which we have assigned to C$_\epsilon$4. However, when DSE transfectoma supernatant was tested in a similar assay, some

reaction was seen against the 51.3 and 84.1C mAb (not shown). It is possible that conformational changes in this mutant have reduced binding to these two anti-IgE mAb, and that these conformational changes in the DSE mutant are more pronounced when the mutant IgE is subjected to high-salt elution from the affinity column. Similarly, in a number of experiments, when fresh DSE supernatants were tested for binding to the Fc$_\epsilon$R on RBL cells using the adsorption assay, some binding to the Fc$_\epsilon$R was detected. Purified fractions of this mutant, eluted with 5 M MgCl$_2$, consistently failed to bind. Thus, the effects of the deletion on the binding to the 84.1C and 51.3 anti-IgE mAb, and on the binding to the Fc$_\epsilon$R are likely to be conformational, rather than due to the elimination of the binding site. We have previously shown [7, 8] that the 51.3, 95.3 and 84.1C anti-IgE mAb are sensitive to IgE conformation and are unable to bind to denatured or reduced IgE.

The anti-IgE antibodies have previously been analyzed in inhibition studies to determine the relationship of the epitopes they recognize to the binding site for IgE. The 51.3 antibody inhibits poorly the binding of IgE to its receptor and is able to bind to IgE receptor complexes [7]. Thus, it seems that this antibody recognizes an epitope which is not closely related to the receptor binding site. This is consistent with our conclusion that the epitope recognized by this antibody is on the C$_\epsilon$4 domain. The 95.3 anti-IgE mAb inhibits the binding of IgE to its Fc$_\epsilon$R but can still bind to cell-bound IgE [7]. Thus the epitope recognized by this antibody is related, but not identical to the receptor binding site. This is also apparent from the fact that the PHS point mutant has lost, entirely, the epitope recognized by 95.3, but is still able to bind to the Fc$_\epsilon$R on mast cells, albeit with reduced affinity. The reduced ability of the PHS mutant to bind to the Fc$_\epsilon$R on RBL cells may be due to a direct effect of the proline to histidine mutation in destabilizing the IgE-Fc$_\epsilon$R interaction, or could be due to conformational changes caused by this mutation which are propagated to distal sites on the C$_\epsilon$3 domain. While more mutants will be required in order to distinguish between these possibilities, it is clear that the 95.3 binding site can be totally abolished with only a moderate effect on Fc$_\epsilon$R binding.

The 84.1C anti-IgE mAb inhibits the binding of IgE to the Fc$_\epsilon$R and does not recognize cell-bound IgE [8]. Thus, it seems likely that this antibody recognizes an epitope very closely related to the binding site for the Fc$_\epsilon$R. It will be interesting to see whether among further IgE point mutants which we are currently producing, one will be found which looses both the ability to bind to the Fc$_\epsilon$R and to the 84.1C anti-IgE mAb. A good candidate for such a mutation may be a site on the "E-peptide-3" produced by Liu et al. [5]. Antibodies against this C$_\epsilon$3 peptide behave like our 84.1C mAb in that they bind to free, but not Fc$_\epsilon$R-bound IgE, and thus seem to cross-inhibit the IgE-Fc$_\epsilon$R interaction.

By combining the above data, a few conclusions may be reached. We have mapped to the C$_\epsilon$3 domain the 84.1C anti-IgE mAb most closely related to the IgE-Fc$_\epsilon$R interaction site. This determinant is not included within the P129 peptide site described by Burt and Stanworth [2]. A single proline to histidine substitution in the region spanned by the P129 peptide leads to the loss of recognition by the 95.3 mAb, which recognizes an epitope that is related, but not identical to the Fc$_\epsilon$R binding site. However, such a mutant (PHS) retains full recognition by the 84.1C anti-IgE mAb, and exhibits only two-fold reduction in binding to the Fc$_\epsilon$R. Thus, the Fc$_\epsilon$R binding

site is probably located in the $C_\epsilon 3$ domain, but not in the region encompassed by P129. The $C_\epsilon 4$ domain seems to be required for the formation of the H_2L_2 tetramer and, though not directly involved in $Fc_\epsilon R$ binding, may play a role in stabilizing the conformation of IgE required for binding to the FcR. The generation of more IgE deletion and point mutants, together with mouse/human exon shuffling experiments (manuscript in preparation) should allow the precise identification of sites on the IgE molecule required for dimerization, binding to mAb and binding to the high-affinity $Fc_\epsilon R$ on mast cells.

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